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Inhibition of N-type Ca^{2+} channels ameliorates an imbalance in cardiac autonomic nerve activity and prevents lethal arrhythmias in mice with heart failure

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Short title: N-type Ca2+ channel inhibition on lethal arrhythmias

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Abstract

Aims__ Dysregulation of autonomic nervous system activity can trigger ventricular arrhythmias and sudden death in patients with heart failure. N-type Ca^{2+} channels (NCCs) play an important role in sympathetic nervous system activation by regulating the calcium entry that triggers release of neurotransmitters from peripheral sympathetic nerve terminals. We have investigated the ability of NCC blockade to prevent lethal arrhythmias associated with heart failure.

Methods and Results__ We compared the effects of cilnidipine, a dual N- and L-type Ca^{2+} channel blocker, with those of nitrendipine, a selective L-type Ca^{2+} channel blocker, in transgenic mice expressing a cardiac-specific, dominant-negative form of neuron-restrictive silencer factor (dnNRSF-Tg). In this mouse model of dilated cardiomyopathy leading to sudden arrhythmic death, cardiac structure and function did not significantly differ among the control, cilnidipine and nitrendipine groups. However, cilnidipine dramatically reduced arrhythmias in dnNRSF-Tg mice, significantly improving their survival rate and correcting the imbalance between cardiac sympathetic and parasympathetic nervous system activity. A β-blocker, bisoprolol, showed similar effects in these mice. Genetic titration of NCCs, achieved by crossing dnNRSF-Tg mice with mice lacking *CACNA1B*, which encodes the α1 subunit of NCCs, improved survival rate. With restoration of cardiac autonomic balance, dnNRSF-Tg;$CACNA1B^{+/-}$ mice showed fewer malignant arrhythmias than dnNRSF-Tg;$CACNA1B^{+/+}$ mice.

Conclusions__ Both pharmacological blockade of NCCs and their genetic titration improved cardiac autonomic balance and prevented lethal arrhythmias in a mouse model of dilated cardiomyopathy and sudden arrhythmic death. Our findings suggest NCC blockade is a potentially useful approach to preventing sudden death in patients with heart failure.

Key words: ion channel; nervous system, autonomic; heart failure; arrhythmia; N-type Ca^{2+} channel

1. Introduction

Approximately 50% of deaths among patients with heart failure are classified as sudden death, mainly caused by lethal arrhythmias[1]. Despite recent progress, pharmacological interventions for the treatment and prevention of lethal arrhythmias associated with chronic heart failure remain unsatisfactory. Nonetheless, it is anticipated that a better understanding of the molecular basis of arrhythmicity in failing hearts will enable identification of therapeutic targets that can serve as the basis for the development of new pharmacological treatments.

Autonomic dysregulation leading to increased sympathetic nerve activity and decreased parasympathetic nerve activity contributes to the increased arrhythmicity seen in patients with chronic heart failure[2, 3]. N-type voltage-dependent Ca^{2+} channels (NCCs), encoded by the *CACNA1B* (α1B subunit) gene, are predominantly localized in the nervous system, where they play a pivotal role in modulating a variety of neuronal functions, including neurotransmitter release at sympathetic nerve terminals[4-6]. Mice lacking *CACNA1B* show functional deterioration of their sympathetic nervous system[7], and the ability of NCC blockade to prevent malignant arrhythmias and sudden death associated with heart failure remains unevaluated.

We previously reported that transgenic mice cardiac-selectively expressing a dominant-negative form of neuron-restrictive silencer factor (NRSF, also called REST) (dnNRSF-Tg), a transcriptional repressor important for regulation of the fetal cardiac gene program, showed progressive cardiomyopathy and sudden arrhythmic death beginning at about 8 weeks of age[8]. We have also reported several abnormalities in cardiac electrophysiological properties and ion channel expression in these dnNRSF-Tg mice[9, 10]. The dnNRSF-Tg hearts showed increased expression of fetal type ion channel genes, including *CACNA1H*, which encodes the T-type Ca^{2+} channel (TCC) α1 subunit, and a corresponding increase in $I_{ca,T}$ amplitude[8]. In that earlier study, we demonstrated that TCC blockade could prevent sudden death in dnNRSF-Tg mice by both restoring the normal electrophysiology of ventricular myocytes and correcting the cardiac autonomic dysfunction observed in dnNRSF-Tg mice[11]. Because TCC expression, and thus functional TCC currents, is increased in the myocardium of dnNRSF-Tg mice, TCC blockade directly affects the electrophysiological properties of ventricular myocytes in dnNRSF-Tg mice. On the other hand, the impact of modulating autonomic nervous system balance on the incidence of lethal arrhythmias in dnNRSF-Tg mice remains unclear.

Pharmacological blockade or genetic deletion of NCCs reportedly alters autonomic activity in both human patients and animal models[7, 12, 13]. On the other hand, little or no NCC expression has been detected in the ventricular myocardium. Therefore, to evaluate the extent to which correcting the autonomic imbalance prevents the lethal arrhythmias

associated with heart failure, we assessed the effects of pharmacological blockade of NCCs and their genetic titration on arrhythmicity and sudden death in dnNRSF-Tg mice. Our findings demonstrate the importance of an imbalance between sympathetic and parasympathetic nerve activities in the generation of lethal arrhythmias in failing hearts, and suggest restoring autonomic nervous system balance through NCC inhibition can be an effective approach to preventing sudden arrhythmic death associated with heart failure.

2. Methods

An expanded Methods section is available in Supplemental Material online.

2.1 Animal experiments

The animal care and all experimental protocols were reviewed and approved by the Animal Research Committee at Kyoto University Graduate School of Medicine, and conformed to the US National Institute of Health Guide for the Care and Use of Laboratory Animals. Beginning at 8 weeks of age, dnNRSF-Tg mice were left untreated (control) or were treated for 24 weeks with cilnidipine (10 mg/kg/day P.O.) or nitrendipine (10 mg/kg/day P.O.). The drug dosages were chosen based on earlier reports and our preliminary studies[14, 15]. Cilnidipine was supplied by Mochida Pharmaceutical Co, Ltd. (Tokyo, Japan). Nitrendipine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bisoprolol was supplied by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). Cilnidipine exerts a much more potent inhibitory effect on N-type Ca^{2+} currents than does nitrendipine, which has little effect on N-type Ca^{2+} currents, particularly under conditions in which L-type Ca^{2+} current inhibition is comparable between the two drugs[16, 17]. We then selected the doses of both drugs that similarly and minimally affected blood pressure. In another experiment, dnNRSF-Tg mice were bred with CACNA1B heterozygous knockout mice to obtain dnNRSF-Tg;CACNA1B$^{+/-}$ mice and control dnNRSF-Tg;CACNA1B$^{+/+}$ littermates. CACNA1B$^{+/-}$ mice were described in an earlier report[7]. For the isolation and analysis of hearts, mice were anesthetized with 3.0 % of isoflurane and sacrificed by cervical dislocation.

2.2 Statistical Analysis

Data are presented as means ± standard errors of the mean (SEM) unless indicated otherwise. Survival was analyzed using the Kaplan-Meier method with the log-rank test. Comparisons among multiple groups were made using ANOVA with post hoc Fisher's tests, except for numbers of arrhythmias. Values of $p<0.05$ were considered significant. Numbers of arrhythmias between two groups were analyzed using the Mann-Whitney test. Values of $p<0.05$ were considered significant. Numbers of arrhythmias among four groups were analyzed using Kruskal-Wallis nonparametric ANOVA followed by the Bonferroni correction. Values of $p<0.0083$ were considered significant in that analysis.

3. Results

3.1 The dual N- and L-type Ca^{2+} channel blocker cilnidipine improves survival among dnNRSF-Tg mice without affecting cardiac structure or function

We initially confirmed that there is little expression of *CACNA1B*, encoding the α1 subunit of NCCs, in either wild-type or dnNRSF-Tg hearts, which is in contrast to its obvious expression in brain (*Figure 1A*). On the other hand, we detected substantially greater ventricular expression of *CACNA1H*, encoding the α1 subunit of TCCs, and *CACNA1C*, encoding the α1 subunit of L-type Ca^{2+} channels (*Figure 1B*). Although ventricular expression of *CACNA1B* is increased in dnNRSF-Tg hearts, probably due to the presence of NRSF-binding element in the gene, the levels are still lower than those of *CACNA1H* in wild-type hearts, where no functional T-type Ca^{2+}currents are detected[11, 18]. To evaluate the potential therapeutic effect of modulating autonomic nervous system activity through NCC blockade on the development of malignant arrhythmias and sudden death in dnNRSF-Tg mice, we administered subpressor doses of cilnidipine, a dual N- and L-type dihydropyridine Ca^{2+} channel blocker, or nitrendipine, a more L-type-selective dihydropyridine Ca^{2+} channel blocker, to dnNRSF-Tg mice for 24 weeks, beginning when they were 8 weeks of age. Under our experimental conditions, systolic blood pressures and heart rates did not differ among the control, cilnidipine and nitrendipine groups of dnNRSF-Tg mice, though blood pressures were slightly lower and heart rates were significantly slower in dnNRSF-Tg mice than in untreated WT mice, as previously reported (Systolic blood pressure: WT, 101.40±1.48; Tg, 96.0±1.75; Tg+cilnidipine, 96.67±1.64; Tg+nitrendipine, 95.47±1.92 mmHg. Heart rates: WT, 682.3±27; dnNRSF-Tg, 590.6±10.9; Tg+cilnidipine, 567.13±17.58; Tg+nitrendipine, 568.8±11.07 /min) (*Figure 1C and D*)[8]. We found that cilnidipine dramatically improved the survival rate among dnNRSF-Tg mice, as compared to mice treated with nitrendipine or untreated control (*Figure 1E*). Although heart-to-body weight ratios were higher in dnNRSF-Tg than WT mice, as reported previously[8], heart-to-body weight ratios did not significantly differ among the control, cilnidipine and nitrendipine groups of dnNRSF-Tg mice (WT, 4.08±0.31; Tg, 5.94±0.24; Tg+cilnidipine, 5.61±0.48; Tg+nitrendipine, 5.94±0.36 mg/g) (*Figure 2A*). Lung-to-body weight ratios also did not differ among these three groups (WT, 5.28±0.37; Tg, 6.07±0.22; Tg+cilnidipine, 5.93±0.79; Tg+nitrendipine, 5.9±0.29 mg/g) (*Figure 2B*). In addition, histological analyses, including determination of the %fibrotic area, and echocardiographic analyses also showed no significant differences among these three groups (*Figure 2C, D, E and F, and Table 1*). By contrast, the echocardiography and histology showed that, as compared to untreated WT mice, left ventricular systolic function was diminished and %fibrotic area was increased in dnNRSF-Tg mice, as reported previously (*Figure 2C, D, E and F, and Table 1*)[8]. Consistent with these findings, there was no significant difference in the expression of two cardiac stress marker genes, *ANP* and *SERCA2*,

among the three groups, whereas their expression did differ between untreated WT mice and dnNRSF-Tg mice, as described previously (*Figure 2G and H*)[8].

Expression of the fibrosis-related genes *Col1a1*, *Col3a1* and *FN1*, encoding collagen type1α1, collagen type3α1 and fibronectin 1, respectively, was not affected by the drug treatments (*Supplemental Figure S1A, B and C*). Expression of genes encoding the fetal-type ion channels *CACNA1H*, *HCN2* and *HCN4* was higher in untreated dnNRSF-Tg ventricles than control WT ventricles, as reported previously, and cilnidipine did not affect expression these genes in dnNRSF-Tg ventricles (*Supplemental Figure S1D,E and F*). Collectively, all of these data indicate that cilnidipine suppresses sudden death in dnNRSF-Tg mice without significantly affecting cardiac structure or function.

3.2 Cilnidipine improves cardiac autonomic nervous system function and reduces arrhythmicity in dnNRSF-Tg mice

We hypothesized that correcting autonomic balance through NCC blockade reduces arrhythmogenicity, thereby improving survival among dnNRSF-Tg mice. HRV is a widely accepted index of cardiac autonomic nervous system activity[19]. Earlier frequency domain analysis of HRV revealed that patients with severe heart failure show a progressive reduction in power in both the low frequency (LF) and high frequency (HF) ranges[19], and that reduction in the LF power range is a significant predictor of sudden cardiac death in patients with heart failure[20]. We used HRV as an index to evaluate cardiac autonomic function in WT and dnNRSF-Tg mice, and examined the effects of cilnidipine on HRV[19]. In mice, HRV predominantly correlates with parasympathetic activity[21]. As we showed previously, both the LF and HF powers averaged over 24 h in dnNRSF-Tg mice (LF, 1.228±0.198; HF, 0.823±0.186 msec^2) were markedly lower than in WT mice (LF, 4.331±0.706; HF, 2.412±0.089 msec^2), indicating a general reduction in parasympathetic activity in dnNRSF-Tg mice (*Figure 3A and B*). Cilnidipine dramatically increased the power in both the LF and HF ranges of HRV (LF, 3.308±0.338; HF, 2.228±0.283 msec^2), whereas nitrendipine had little effect on HRV (LF, 0.538±0.447; HF, 1.383±0.57 msec^2) (*Figure 3A and B*). We also found that urinary excretion of norepinephrine, which is indicative of the level of sympathetic nerve activity, was significantly higher in dnNRSF-Tg than WT mice, and that norepinephrine excretion was significantly reduced only by cilnidipine (WT, 0.09±0.02; Tg, 0.33±0.04; Tg+cilnidipine, 0.15±0.03; Tg+nitrendipine, 0.32±0.1 μg/day) (*Figure 3C*).

We next used an implanted telemetric monitoring system to examine the effects of cilnidipine and nitrendipine on electrocardiographic parameters in dnNRSF-Tg mice. We found that only cilnidipine significantly suppressed the number of premature ventricular contractions (PVCs) in dnNRSF-Tg hearts (WT, 0±0; dnNRSF-Tg, 502.66±305.69; dnNRSF-Tg+cilnidipine, 1.0±0.66; dnNRSF-Tg+nitrendipine, 326.17±147.24 /hours) (*Figure*

3D). More importantly, it dramatically reduced the number of episodes of ventricular tachycardia (VT) (WT, 0±0; dnNRSF-Tg, 14.92±4.95; dnNRSF-Tg+cilnidipine, 0.06±0.06; dnNRSF-Tg+nitrendipine, 12.75±5.16 /hours) (*Figure 3E and Supplemental Figure S2A and B*). These lines of evidence suggest that by restoring autonomic nervous system balance, cilnidipine reduces the incidence of lethal arrhythmias in dnNRSF-Tg mice.

3.3 β-adrenergic receptor blockade prevents lethal arrhythmias and sudden death in dnNRSF-Tg mice

To verify the importance of correcting autonomic nervous system imbalance for the prevention of lethal arrhythmias and sudden death in dnNRSF-Tg mice, irrespective of effects on structural remodeling, we examined the effects of treating these mice with a β-adrenergic receptor blocker. We administered a subpressor dose of the lipophilic β-adrenergic receptor blocker bisoprolol (1 mg/kg/day P.O.) to WT and dnNRSF-Tg mice. Although systolic blood pressures did not differ between untreated control and bisoprolol-treated mice (untreated WT, 107.5±1.6; WT+bisoprolol, 108.0±1.2; untreated Tg, 98.6±2.0; Tg+bisoprolol, 98.6±1.7 mmHg) (*Figure 3F*), heart rates were significantly slower in bisoprolol-treated than untreated WT and dnNRSF-Tg mice (untreated WT, 697.8±8.3; WT+bisoprolol, 604.7±38.3; Tg, 601.6±10.1; Tg+bisoprolol, 558.6±12.0 /min) (*Figure 3G*). At the dose tested, bisoprolol also did not affect cardiac systolic function assessed echocardiographically in dnNRSF-Tg mice (LVDd: WT, 3.3±0.2; WT+bisoprolol, 3.2±0.1; Tg, 3.9±0.1; Tg+bisoprolol, 3.9±0.1 mm. EF: WT, 84.5±4.0; WT+bisoprolol, 83.0±1.5; Tg, 46.0±1.6; Tg+bisoprolol, 51.5±2.7 %) (*Figure 3H and I*). On the other hand, bisoprolol significantly restored power in both the LF and HF ranges of HRV (LF, untreated WT, 5.19±0.37; Tg, 1.36±0.14; Tg+bisoprolol, 3.34±0.39 $msec^2$. HF, untreated WT, 2.12±0.24; Tg, 0.86±0.12; Tg+bisoprolol, 1.62±0.22 $msec^2$) (*Figure 3J and K*) and reduced the incidence of VPCs and VTs in those mice (VPC: Tg, 408.3±122.9; Tg+bisoprolol 98.9±42.2 /hours; VT: Tg, 28.2±12.1; Tg+bisoprolol 7.6±1.7 /hours) (*Figure 3L and M)*. As a result, bisoprolol significantly improved survival rates among dnNRSF-Tg mice (*Figure 3N*). These results strongly support our finding that imbalance of autonomic nervous system activities is critically involved in the occurrence of sudden arrhythmic death in dnNRSF-Tg mice.

3.4 Genetic titration of NCC improves survival among dnNRSF-Tg mice

To further confirm the benefit of NCC inhibition for prevention of sudden death in dnNRSF-Tg mice, we next genetically titrated NCC expression by crossing dnNRSF-Tg mice with mice lacking *CACNA1B*, encoding the α1B subunit of NCC. Because the $CACNA1B^{-/-}$ genotype has a high incidence of early mortality from an as yet unknown cause, we compared the phenotypes of dnNRSF-Tg;$CACNA1B^{+/+}$ mice with those of dnNRSF-Tg;$CACNA1B^{+/-}$ mice, in which NCC expression is reduced to about 52.9% of that

in dnNRSF-Tg;CACNA1B$^{+/+}$ mice (*Figure 4A*). The gross appearance of CACNA1B$^{+/-}$ mice is normal, and they show no early mortality. Systolic blood pressures in dnNRSF-Tg;CACNA1B$^{+/-}$ and dnNRSF-Tg;CACNA1B$^{+/+}$ mice did not significantly differ, but were mildly lower than in control WT (CACNA1B$^{+/+}$) mice (WT, 101.25±7.26; CACNA1B$^{+/-}$, 91.25±2.78; dnNRSF-Tg, 92±4.38; dnNRSF-Tg;CACNA1B$^{+/-}$, 89.25±2.14 mmHg) (*Figure 4B*). Similarly, heart rates did not differ between dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice, although they were slower in dnNRSF-Tg;CACNA1B$^{+/+}$ than control WT mice, as reported previously (WT, 632.25±26.36; CACNA1B$^{+/-}$, 594±33.39; dnNRSF-Tg, 515.25±14.71; dnNRSF-Tg;CACNA1B$^{+/-}$, 521.5±23.32 /min) (*Figure 4C*)[8]. Body weights were comparable between the two dnNRSF-Tg groups (CACNA1B$^{+/+}$, 31.08±1.11; CACNA1B$^{+/-}$, 29.53±1.37; dnNRSF-Tg;CACNA1B$^{+/+}$, 28.86±1.19; dnNRSF-Tg;CACNA1B$^{+/-}$, 27.41±1.09 g) (*Figure 4D*), but heart-to-body weight ratios were higher in dnNRSF-Tg;CACNA1B$^{+/+}$ than WT (CACNA1B$^{+/+}$) mice and were significantly lower in dnNRSF-Tg;CACNA1B$^{+/-}$ than dnNRSF-Tg;CACNA1B$^{+/+}$ mice (CACNA1B$^{+/+}$, 4.44±0.04; CACNA1B$^{+/-}$, 4.51±0.14; dnNRSF-Tg; CACNA1B$^{+/+}$, 5.68±0.21; dnNRSF-Tg;CACNA1B$^{+/-}$, 4.86±0.18 mg/g) (*Figure 4E*). Lung-to-body weight ratios were comparable between the two dnNRSF-Tg groups (CACNA1B$^{+/+}$, 5.06±0.22; CACNA1B$^{+/-}$, 4.68±0.96; dnNRSF-Tg; CACNA1B$^{+/+}$, 5.41±0.09; dnNRSF-Tg;CACNA1B$^{+/-}$, 5.52±0.26 mg/g) (*Figure 4F*). Echocardiographic analysis showed that left ventricular diastolic dimension (LVDd) was higher in dnNRSF-Tg than WT mice, while ejection fraction (EF) was lower in dnNRSF-Tg;CACNA1B$^{+/+}$ than WT mice, as was reported previously (*Figure 5A and B*)[8]. In addition, LVDd was lower and EF was higher in dnNRSF-Tg;CACNA1B$^{+/-}$ than dnNRSF-Tg;CACNA1B$^{+/+}$ mice (*Figure 5A and B, and Table 1*).

Histological analysis revealed no significant difference between dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice, although %fibrotic area showed a trend toward being smaller in dnNRSF-Tg;CACNA1B$^{+/-}$ than dnNRSF-Tg;CACNA1B$^{+/+}$ mice (*Figure 5C and D*). Expression of the fibrosis-related genes *Col1a1*, *Col3a1* and *FN1* did not significantly differ between dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice (*Supplemental Figure S3A, B and C*), though there was a significant difference in the expression of *ANP* and *SERCA2* between these two genotypes (*Figure 5E and F*). Genetic reduction in *CACNA1B* also significantly affected expression of *CACNA1H* and *HCN2*, but not *HCN4*, in dnNRSF-Tg ventricles (*Supplemental Figure S3D, E and F*). All of these data demonstrate that genetic reduction of *CACNA1B* tends to ameliorate impaired cardiac function and pathological remodeling in dnNRSF-Tg mice. Furthermore, survival among dnNRSF-Tg;CACNA1B$^{+/-}$ mice was dramatically and significantly better than among control dnNRSF-Tg;CACNA1B$^{+/+}$

mice (*Figure 6A*), demonstrating that reduction of NCC prevents sudden arrhythmic death in dnNRSF-Tg mice.

3.5 Reducing *CACNA1B* expression improves autonomic function and decreases the occurrence of arrhythmias in dnNRSF-Tg mice

We also assessed autonomic nervous system activity in dnNRSF-Tg;CACNA1B$^{+/-}$ and dnNRSF-Tg;CACNA1B$^{+/+}$ mice. In HRV analyses, the reductions in LF and HF power otherwise seen in dnNRSF-Tg;CACNA1B$^{+/+}$ mice (LF, 1.288±0.16; HF, 1.168±0.108 msec^2) were significantly ameliorated in dnNRSF-Tg;CACNA1B$^{+/-}$ mice (LF, 3.54±0.47; HF, 3.075±0.468 msec^2), indicating a restoration of parasympathetic activity through reduction of NCC function (*Figure 6B and C*). In addition, we found that the increase in urinary excretion of norepinephrine seen in dnNRSF-Tg;CACNA1B$^{+/+}$ mice (0.428±0.07 μg/day) was significantly ameliorated in dnNRSF-Tg;CACNA1B$^{+/-}$ mice (0.154±0.05 μg/day) (*Figure 6D*). Finally, evaluation of arrhythmicity revealed that the incidences of both PVCs and VT were significantly lower in dnNRSF-Tg;CACNA1B$^{+/-}$ than dnNRSF-Tg;CACNA1B$^{+/+}$ mice (VPC: WT, 0±0; CACNA1B$^{+/-}$, 0±0; dnNRSF-Tg, 239.08±27.93; dnNRSF-Tg;CACNA1B$^{+/-}$, 3.21±3.21. VT: WT, 0±0; CACNA1B$^{+/-}$, 0±0; dnNRSF-Tg, 41.3±12.69; dnNRSF-Tg;CACNA1B$^{+/-}$, 0.36±0.36 /hours,) (*Figure 6E and F*). These results demonstrate that genetic titration of *CACNA1B*, encoding NCC, corrected an imbalance between sympathetic and parasympathetic nervous system activities, which, at least in part, contributes to reducing malignant arrhythmias in dnNRSF-Tg mice in a manner similar to pharmacological NCC blockade.

4. Discussion

Autonomic dysregulation leading to increased sympathetic nerve activity and reduced parasympathetic nerve activity is reportedly associated with the increased arrhythmicity seen in patients with chronic heart failure[2, 22, 23]. NCCs play a major role in the release of norepinephrine at sympathetic nerve terminals[7, 24]. Consequently, mice lacking *CACNA1B*, the gene encoding the α1 subunit of NCCs, exhibit a significantly impaired positive inotropic response[7]. In the present study, we found that pharmacological blockade of NCCs or their genetic titration improved the balance between sympathetic and parasympathetic nerve activities and prevented the sudden death and arrhythmicity otherwise seen in dnNRSF-Tg mice, a mouse model of sudden arrhythmic death associated with cardiac dysfunction[8]. The mode of death in these model mice is sudden and without overt edema, pleural effusion or apparent lung congestion, and all the telemetry data obtained at the time of death indicates VT/VF to be the cause[8]. Moreover, in an earlier study we found that systemic administration of isoproterenol induced VT more frequently in dnNRSF-Tg than WT mice[11]. Conversely, administration of a β-blocker led to a significant reduction in the incidence of sudden death among dnNRSF-Tg mice under conditions in which cardiac systolic function and remodeling were not affected (*Figure 3H-N*). These findings suggest NCC blockade or genetic titration of NCC reduces the likelihood of sudden arrhythmic death, thereby improving survival.

Pharmacological interventions that reduce cardiac sympathetic activity have been shown to protect against arrhythmias[25], while interventions that stimulate cardiac sympathetic activity provoke malignant arrhythmias[2, 26]. In patients with heart failure, β-adrenoreceptor blockade reduces the incidence of sudden death[27, 28]; however, β-blockers are not completely protective, and mortality remains high among patients with cardiac dysfunction, despite optimal β-blocker therapy[27, 28]. It is therefore necessary to find other approaches to modulate sympathetic or parasympathetic activity. In that context, a clinical trial testing the effect of central modulation of sympathetic activity using moxonidine SR in patients with heart failure was terminated early due to an increase in mortality and morbidity in patients receiving the drug[29]. Thus strong central inhibition of the sympathetic nervous system through imidazoline receptor stimulation appears not to protect against lethal arrhythmias. NCCs are localized mainly at peripheral sympathetic nerve terminals, where they regulate the release of neurotransmitters (e.g., catecholamines), thereby modulating sympathetic activity[4-6]. Our findings suggest that, by correcting their autonomic dysregulation, NCC blockade could be an effective approach to preventing sudden arrhythmic death in patients with heart failure.

Cilnidipine failed to prevent the decline in cardiac function in dnNRSF-Tg mice, whereas genetic titration tended to ameliorate the adverse cardiac remodeling and cardiac dysfunction seen in dnNRSF-Tg mice (*Figure 2A-2H, 4E, 5A-5F and Table 1*). The reasons

for the difference in the effects on cardiac function between cilnidipine and genetic titration of NCCs remain unclear at present. It may be that cilnidipine's ability to block L-type Ca^{2+} channels has a detrimental effect on cardiac function, as L-type Ca^{2+} channel blockers can adversely affect the progression of heart failure[30]. Other possibilities are that the relatively low dose of cilnidipine used in this study was not sufficient to prevent the progression of cardiac dysfunction, though it did prevent lethal arrhythmias, or that the NCC inhibition achieved in CACNA1B$^{+/-}$ mice was more prolonged and more stable than that achieved with cilnidipine, which was not started until the mice were 8 weeks of age. The effects on NCCs expressed in the central nervous system could also differ between cilnidipine and genetic titration, as cilnidipine has little ability to cross the blood-brain barrier[31]. These differences suggest the underlying mechanisms involved in the reduced incidence of lethal arrhythmias and the prolonged survival differ somewhat between cilnidipine treatment and genetic titration of CACNA1B in this study. Cilnidipine treatment, which improved autonomic imbalance and reduced lethal arrhythmias without affecting cardiac remodeling, mainly suppressed the triggering of lethal arrhythmias induced by autonomic imbalance. On the other hand, genetic titration of CACNA1B, which improved autonomic imbalance and also tended to prevent adverse cardiac remodeling, suppressed lethal arrhythmias and improved survival in two ways: it inhibited the triggering of arrhythmias and also suppressed the generation of arrhythmogenic substrates. In both cases, correcting the autonomic imbalance associates with a reduction in the incidence of sudden death attributable to lethal arrhythmias in dnNRSF-Tg. However, because it is not possible to completely exclude the possibility that some dnNRSF-Tg mice (especially older mice) die due to congestive heart failure, irrespective of arrhythmias, there is a possibility that genetic deletion of NCC may also prevent this mode of death in addition to sudden arrhythmic death in dnNRSF-Tg mice through suppression of excessive sympathetic activity.

In the present study, both pharmacological blockade of NCCs and their genetic titration not only repressed sympathetic activity, as demonstrated by a reduction in urinary norepinephrine levels, but also restored parasympathetic activity, as indicated by HRV analyses. The precise mechanism by which NCC inhibition improves parasympathetic activity is not clear at present. However, accumulating data indicate the sympathetic and parasympathetic nervous systems interact via several mechanisms at both the central and peripheral levels of the neuraxis[32]. NCC inhibition-induced reductions in sympathetic activity may affect these interactions, ameliorating the reduction in parasympathetic activity, as was observed in dnNRSF-Tg mice. In humans, cilnidipine reportedly enhances parasympathetic activity in hypertensive patients while exerting a concomitant sympathoinhibitory effect[12, 13]. Moreover, there is now much evidence showing the anti-arrhythmic effects of parasympathetic nervous activation. This suggests that, in addition to a reduction in sympathetic activity, an increase in parasympathetic activity likely

contributes to the protective effects of NCC inhibition observed in this study[27]. Although further investigation is necessary, our study suggests that agents able to selectively block NCCs, could be clinically useful for the prevention of sudden arrhythmic death in patients with heart failure.

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Conflict of interest

None

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Figure Legends

Figure 1. Pharmacological blockade of NCCs by cilnidipine improves survival among dnNRSF-Tg mice. **A**, Relative levels of CACNA1B mRNA in brains (B) from wild-type (WT), kidney (K) from WT, cardiac ventricle (V) from WT and cardiac ventricle (V) from 8-week-old dnNRSF-Tg mice (Tg); levels in cardiac ventricle from WT mice were assigned a value of 1.0. n=3 each for brain, kidney and cardiac ventricle from WT mice and 2 for cardiac ventricle from dnNRSF-Tg mice. **B**, Relative levels of CACNA1B, CACNA1H and CACNA1C mRNA in cardiac ventricle from 8-week-old WT mice and dnNRSF-Tg mice (Tg); levels of CACNA1B mRNA in WT mice were assigned a value of 1.0. n=5 for WT mice and 7 for dnNRSF-Tg. **C** and **D**, Systolic blood pressures (**C**) and heart rates (**D**) in 20-week-old untreated WT, untreated Tg (Tg-cont), cilnidipine-treated Tg (Tg-Cil) and nitrendipine-treated Tg-Nit mice (n=15 each for untreated Tg, Tg-Cil and Tg-Nit, and 10 for untreated WT). ANOVA with post hoc Fisher's tests was used for analysis. *$p<0.05$. N.S.: not significant. **E**, Kaplan-Meyer survival curves for untreated WT, untreated Tg, Cil-treated Tg and Nit-treated Tg over a 24-week drug administration period (from 8 to 32 weeks of age): Log-rank test was used for analysis. *$p<0.05$ (n = 21 for WT, 23 for Tg without drugs, 22 for Tg + Cil, 20 for Tg + Nit). The numbers of mice alive in each group at the end of each period are shown at the bottom of the figure. All data except survival curves are shown as means ± SEM.

Figure 2. Cilnidipine does not affect cardiac structure or function in dnNRSF-Tg mice. **A** and **B**, heart-to-body weight (HW/BW) ratios (**A**) and lung-to-body weight (LungW/BW) ratios (**B**) in 20-week-old untreated WT (WT-cont), untreated Tg (Tg-cont), Cil-treated Tg (Tg-Cil) and Nit-treated Tg (Tg-Nit) mice (n=5 for untreated WT, 4 for Tg-cont, 4 for Tg-Cil and 3 for Tg-Nit). **C**, Histology of hearts from 20-week-old untreated WT, Tg-cont, Tg-Cil and Tg-Nit mice: H-E, hematoxylin-eosin staining; Sirius-red, Sirius-red staining. Scale bars, 100 μm. **D**, %fibrotic area in 20-week-old untreated WT, Tg-cont, Tg-Cil and Tg-Nit mice (n=5 for Tg-cont, n=7 for Tg-Cil). N.S.: not significant. **E** and **F**, Left ventricular end-diastolic dimension (LVDd) (**E**) and ejection fraction (EF) (**F**) assessed echocardiographically in untreated WT, Tg-cont, Tg-Cil and Tg-Nit mice. *$p<0.05$. N.S.: not significant. (n=5 each for untreated WT, Tg-cont and Tg-Cil, n=7 for Tg-Nit). **G** and **H**, Relative levels of ANP (**G**) and SERCA2 (**H**) mRNA in cardiac ventricles from untreated WT, Tg-cont, Tg-Cil and Tg-Nit mice; levels in untreated WT were assigned a value of 1.0. N.S.: not significant. (n=4 each). ANOVA with post hoc Fisher's tests was used for analysis.. All data are shown as means ± SEM.

Figure 3. Cilnidipine restores cardiac autonomic nervous system balance and reduces arrhythmias in dnNRSF-Tg mice. **A** and **B**, Average power of the low frequency (LF) (**A**) and high frequency (HF) (**B**) components of heart rate variability (HRV) recorded over a 24-h period in 20-week-old untreated WT (WT-cont), untreated Tg (Tg-cont), Cil-treated Tg (Tg-Cil) and Nit-treated (Tg-Nit) mice. *p<0.05. N.S.: not significant (n = 5 for WT, 6 for Tg-cont, 8 for Tg-Cil, 6 for Tg-Nit). **C**, Urinary norepinephrine (NE) levels in 20-week-old WT-cont, Tg-cont, Tg-Cil and Tg-Nit mice. *p<0.05. N.S.: not significant (n = 7 for WT, 7 for Tg-cont, 5 for Tg-Cil, 4 for Tg-Nit). **D** and **E**, Numbers of PVC (**D**) and VT (**E**) recorded with a telemetry system in 20-week-old WT-cont, Tg-cont, Tg-Cil and Tg-Nit mice are shown by dot plots. *p<0.0083, N.S.: not significant (n = 5 for WT-cont, 6 for Tg-cont, 8 for Tg-Cil, 6 for Tg-Nit). **F** and **G**, Systolic blood pressures (**F**) and heart rates (**G**) in 20-week-old untreated WT (WT-cont), bisoprolol (Bis)-treated WT (WT-Bis), untreated Tg (Tg-cont) and Bis-treated Tg (Tg-Bis) mice (n=4 for WT-cont, 3 for WT-Bis, 5 for Tg-cont and Tg-Bis). **H** and **I**, Left ventricular end-diastolic dimension (LVDd) (**H**) and ejection fraction (EF) (**I**) assessed echocardiographically in WT-cont, WT-Bis, Tg-cont and Tg-Bis mice. *p<0.05. N.S.: not significant. (n=4 for WT-cont, 3 for WT-Bis, 5 for Tg-cont and Tg-Bis). **J** and **K**, Average power of the low frequency (LF) (**J**) and high frequency (HF) (**K**) components of heart rate variability (HRV) recorded over a 24-h period in 20-week-old WT-cont, Tg-cont and Tg-Bis mice. *p<0.05. N.S.: not significant (n = 4 for WT-cont, 6 for Tg-cont, 7 for Tg-Bis). **L** and **M**, Numbers of PVC (**L**) and VT (**M**) recorded with a telemetry system in 20-week-old Tg-cont and Tg-Bis mice are shown by dot plots. *p<0.05 (n = 6 for Tg-cont, 7 for Tg-Bis). ANOVA with post hoc Fisher's tests was used for analysis, except for numbers of arrhythmias (**D, E, L,** and **M**). Numbers of arrhythmias among the four groups were analyzed using Kruskal-Wallis nonparametric ANOVA followed by the Bonferroni correction (**D** and **E**). Numbers of arrhythmias between two groups were analyzed using non-parametric Mann-Whitney test (**L** and **M**). **N**, Kaplan-Meyer survival curves for untreated Tg and Biso-treated Tg (Tg+Bis) over a 90-days drug administration period (from 12 to 25 weeks of age): Log-rank test was used for the survival analysis. *p<0.05 (n = 15 each). The numbers of mice alive in each group at the end of each period are shown at the bottom of the figure. All data except numbers of arrhythmias and survival curves are shown as means ± SEM.

Figure 4. Effects of genetic titration of *CACNA1B* on hemodynamics and heart size in WT and dnNRSF-Tg mice. **A**, CACNA1B mRNA expression in brains from 8-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$ and CACNA1B$^{-/-}$ mice; the level in CACNA1B$^{+/+}$ brain was assigned a value of 1.0. **B** and **C**, Systolic blood pressures (**B**) and heart rates (**C**) in 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and

dnNRSF-Tg;CACNA1B$^{+/-}$ mice. N.S.: not significant (n = 4 each). **D**, **E** and **F**, body weights (BW)(**D**), heart-to-body weight ratios (HW/BW) (**E**) and lung-to-body weight ratios (LungW/BW) (**F**) in 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice. *p<0.05. N.S.: not significant. (BW and HW/BW: n=4 for CACNA1B$^{+/+}$, 6 for CACNA1B$^{+/-}$, 5 for dnNRSF-Tg;CACNA1B$^{+/+}$, 7 for dnNRSF-Tg;CACNA1B$^{+/-}$. LungW/BW: n=4 for CACNA1B$^{+/+}$, 6 for CACNA1B$^{+/-}$ and dnNRSF-Tg;CACNA1B$^{+/-}$, 5 for dnNRSF-Tg;CACNA1B$^{+/+}$). ANOVA with post hoc Fisher's tests was used for analysis. All data are shown as means ± SEM.

Figure 5. Effect of genetic titration of *CACNA1B* on cardiac structure and function in dnNRSF-Tg mice. **A** and **B**, Left ventricular end-diastolic dimension (LVDd) and ejection fraction (EF) assessed echocardiographically in 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, CACNA1B$^{+/+}$; dnNRSF-Tg and CACNA1B$^{+/-}$; dnNRSF-Tg mice. *p<0.05. (n = 13 for CACNA1B$^{+/+}$, 14 for CACNA1B$^{+/-}$, 11 for dnNRSF-Tg;CACNA1B$^{+/+}$, 15 for dnNRSF-Tg;CACNA1B$^{+/-}$). **C**, Histology of hearts from 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice. H-E, hematoxylin-eosin staining; Sirius-red, Sirius-red staining. Scale bars, 100 μm. **D**, %Fibrotic area in the indicated groups (n=4 for CACNA1B$^{+/+}$, 6 for CACNA1B$^{+/-}$, 5 for dnNRSF-Tg;CACNA1B$^{+/+}$, 7 for dnNRSF-Tg;CACNA1B$^{+/-}$). N.S.: not significant. **E** and **F**, Relative levels of ANP and SERCA2 mRNA in cardiac ventricles from 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice (n = 4 for CACNA1B$^{+/+}$, 6 for CACNA1B$^{+/-}$, 5 for dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$); levels in CACNA1B$^{+/+}$ ventricles were assigned a value of 1.0. *p<0.05. ANOVA with post hoc Fisher's tests was used for analysis. All data are shown as means ± SEM.

Figure 6. Genetic titration of *CACNA1B* restores cardiac autonomic nervous system balance and reduces arrhythmias in dnNRSF-Tg mice. **A**, Kaplan-Meyer survival curves for CACNA1B$^{+/+}$ (1B$^{+/+}$), CACNA1B$^{+/-}$ (1B$^{+/-}$), dnNRSF-Tg;CACNA1B$^{+/+}$ (Tg/1B$^{+/+}$) and dnNRSF-Tg;CACNA1B$^{+/-}$ (Tg/1B$^{+/-}$) mice. Curves cover the span from birth to 32 weeks of age. Log-rank test was used for analysis. *p<0.05 (n=22 for CACNA1B$^{+/+}$, 38 for CACNA1B$^{+/-}$, 20 for dnNRSF-Tg;CACNA1B$^{+/+}$, 42 for dnNRSF-Tg;CACNA1B$^{+/-}$). The numbers of mice alive in each group at the end of each period are shown at the bottom of the figure. **B** and **C**, Average power of the low frequency (LF) (**B**) and high frequency (HF) (**C**) components of heart rate variability (HRV) recorded over a 24-h period in 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice. *p<0.05 (n=5 for CACNA1B$^{+/+}$, 7 for CACNA1B$^{+/-}$, 6 for dnNRSF-Tg;CACNA1B$^{+/+}$,

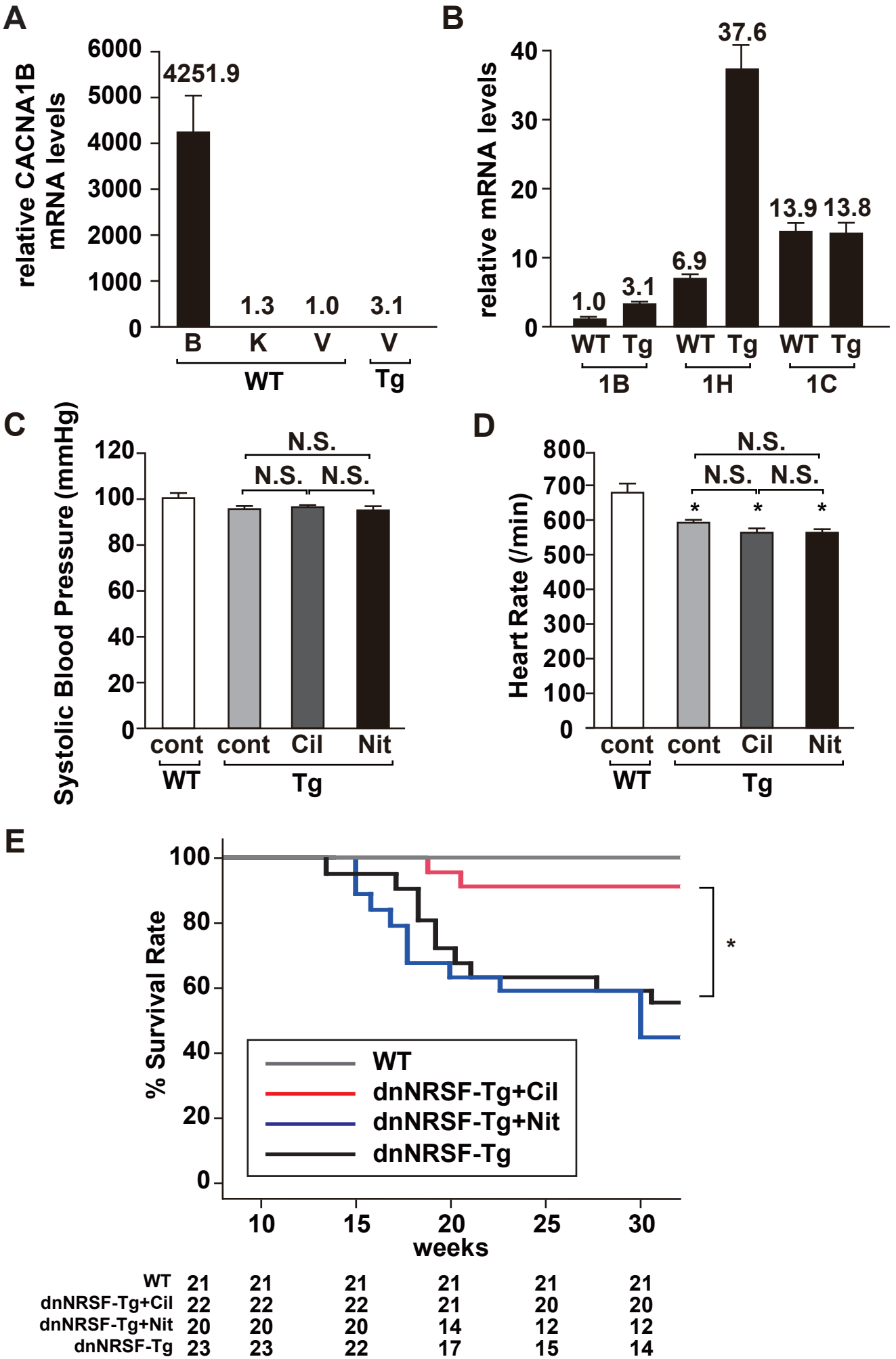
7 for dnNRSF-Tg;CACNA1B$^{+/-}$). **D**, Urinary norepinephrine (NE) levels in 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice. *p<0.05 (n=5 for CACNA1B$^{+/-}$, 6 for CACNA1B$^{+/+}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$). **E** and **F**, Numbers of PVC (**E**) and VT (**F**) recorded using a telemetry system in 20-week-old CACNA1B$^{+/+}$, CACNA1B$^{+/-}$, dnNRSF-Tg;CACNA1B$^{+/+}$ and dnNRSF-Tg;CACNA1B$^{+/-}$ mice are shown by dot plot. *p<0.0083 (n=5 for CACNA1B$^{+/+}$, 7 for CACNA1B$^{+/-}$, 6 for dnNRSF-Tg;CACNA1B$^{+/+}$, 7 for dnNRSF-Tg;CACNA1B$^{+/-}$). All data in **B**-**D** are shown as means ± SEM. ANOVA with post hoc Fisher's tests was used for analysis, except for numbers of arrhythmias (**E** and **F**). Numbers of arrhythmias were analyzed using Kruskal-Wallis nonparametric ANOVA followed by the Bonferroni correction.

Table 1. Echocardiographic parameters in 20-week-old mice.

Pharmacological inhibition				
	WT	dnNRSF-Tg		
	control	cont	Cil	Nit
LVDd (mm)	3.3±0.13	3.9±0.19	4.0±0.11	3.8±0.08
LVDs (mm)	2.1±0.08	3.1±0.17	3.1±0.11	2.9±0.10
IVST (mm)	0.76±0.02	0.72±0.02	0.72±0.02	0.71±0.03
PWT (mm)	0.76±0.02	0.74±0.02	0.76±0.02	0.76±0.03
FS (%)	36.1±2.3	20.3±1.4	23.3±2.7	23.8±2.4
EF (%)	73.2±2.7	49.0±2.3	55.4±4.2	57.0±4.3
Genetic titration				
			dnNRSF-Tg	
	$1B^{+/+}$	$1B^{+/-}$	$1B^{+/+}$	$1B^{+/-}$
LVDd (mm)	3.2±0.10	3.3±0.08	4.1±0.12	3.3±0.07*
LVDs (mm)	2.2±0.12	2.2±0.06	3.2±0.13	2.3±0.08*
IVST (mm)	0.66±0.01	0.68±0.02	0.66±0.02	0.69±0.02
PWT (mm)	0.68±0.02	0.67±0.02	0.66±0.02	0.68±0.02
FS (%)	31.8±1.8	33.1±1.9	20.4±1.3	30.4±1.3*
EF (%)	66.4±2.4	68.9±2.6	49.0±2.4	64.3±1.8*

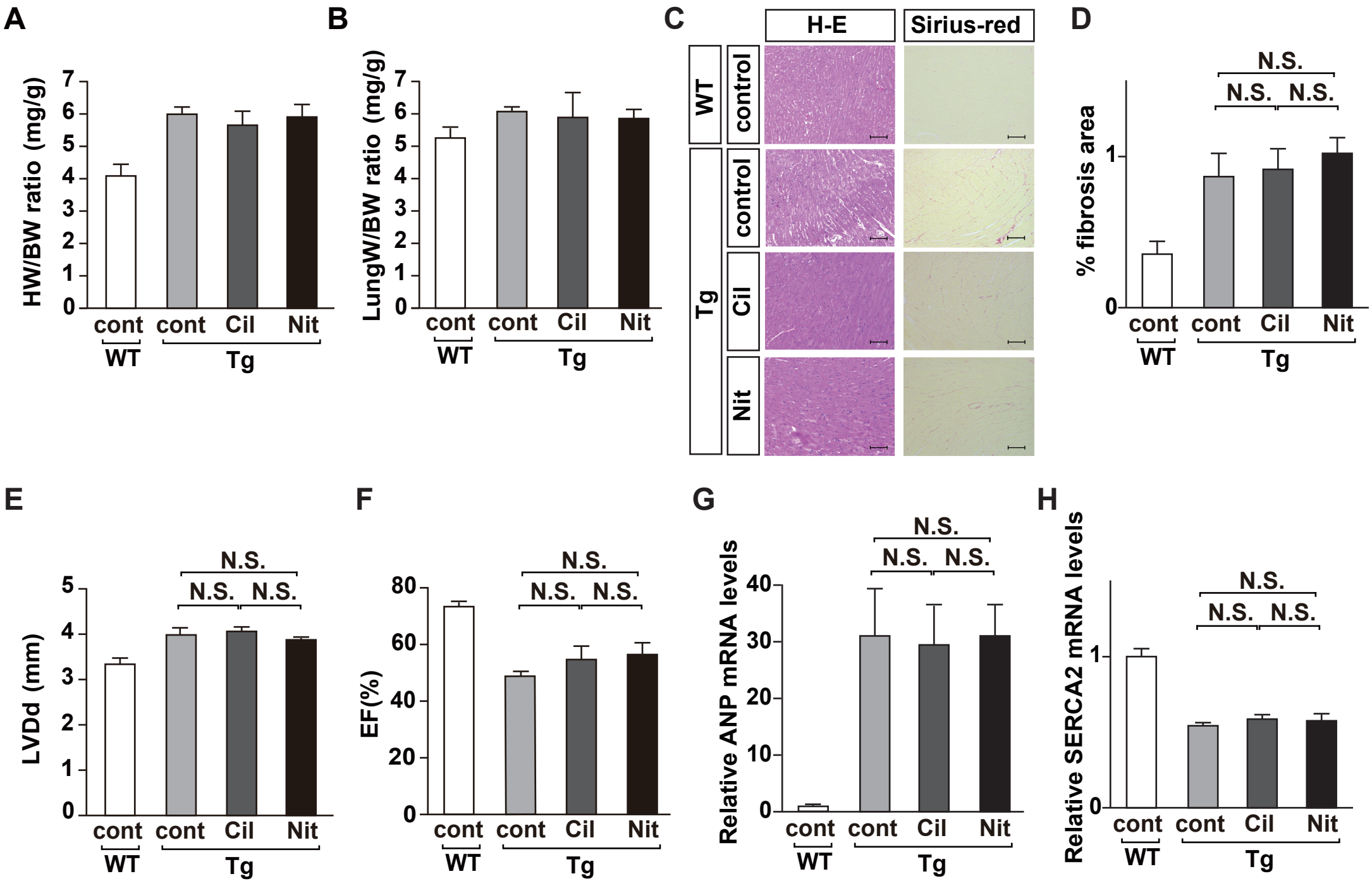
Values are means ± SEM. Cil, cilnidipine; Nit, nitrendipine; $1B^{+/+}$, $CACNA1B^{+/+}$; $1B^{+/-}$, $CACNA1B^{+/-}$; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; FS, fractional shortening; IVST, intraventricular septum wall thickness; PWT, posterior wall thickness. Numbers of mice tested in the pharmacological inhibition study are as follows: n = 5 for WT, untreated dnNRSF-Tg and Cil-treated dnNRSF-Tg;7 for Nit-treated dnNRSF-Tg (upper panel). Numbers of mice tested in the genetic titration study are as follows: n = 13 for $1B^{+/+}$, 14 for $1B^{+/-}$, 11 for dnNRSF-Tg;$1B^{+/+}$ and 15 for dnNRSF-Tg; $1B^{+/-}$ (lower panel). ANOVA with post hoc Fisher's tests was used for the analysis. *$p<0.05$ vs. dnNRSF-Tg;$1B^{+/+}$.

Figure 1



WT	21	21	21	21	21	21
dnNRSF-Tg+Cil	22	22	22	21	20	20
dnNRSF-Tg+Nit	20	20	20	14	12	12
dnNRSF-Tg	23	23	22	17	15	14

Figure 2



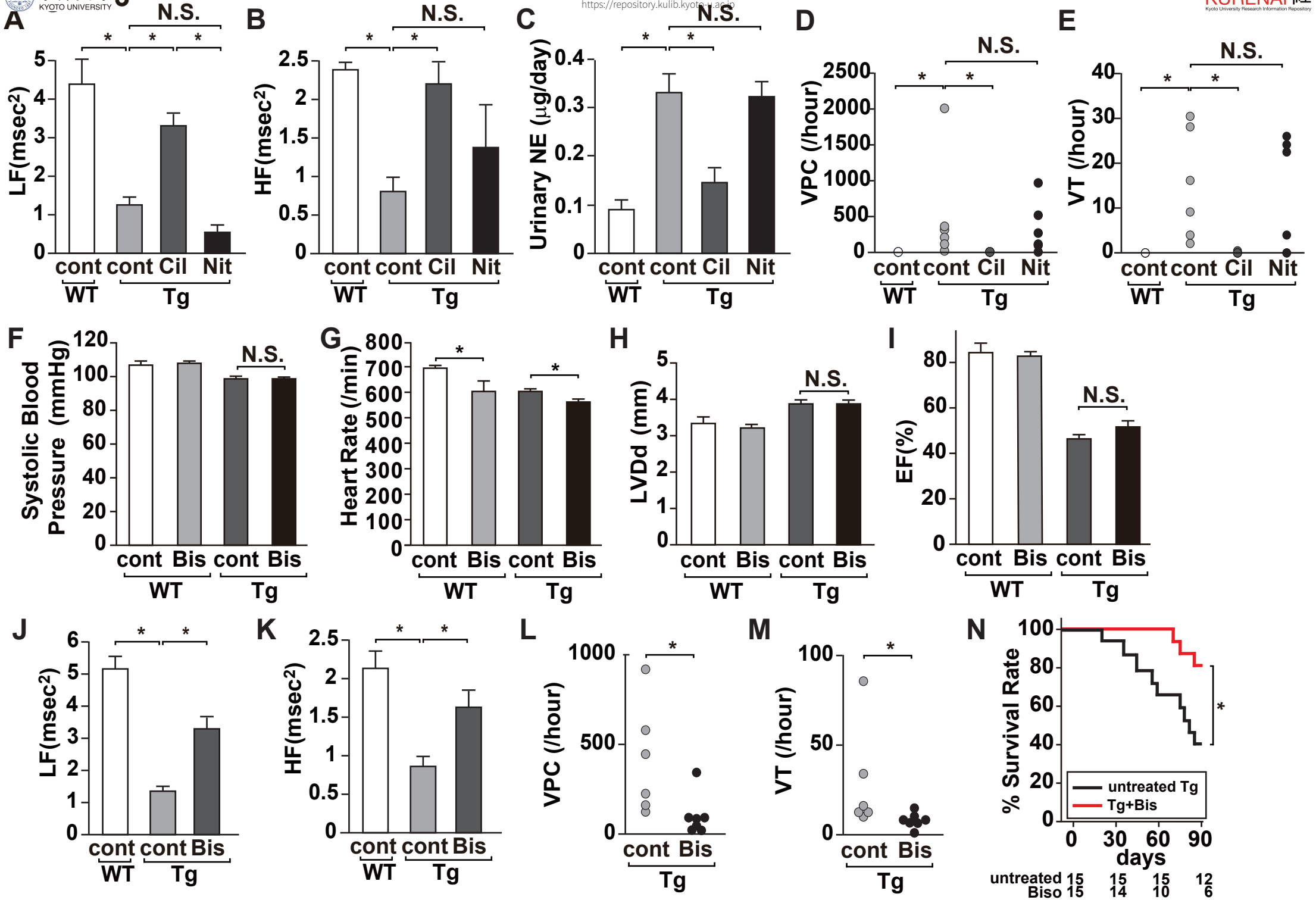


Figure 4

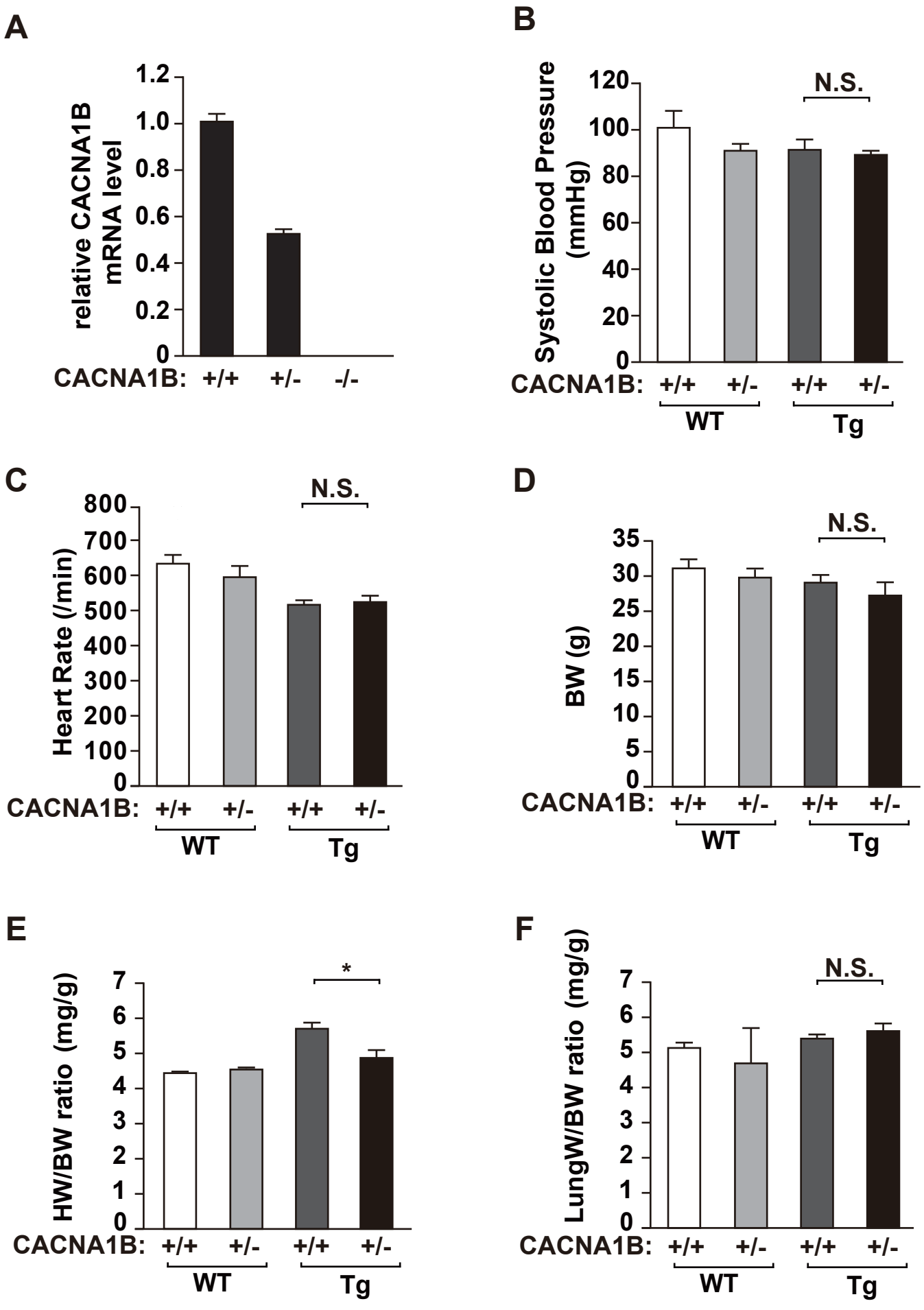


Figure 5